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ORIGINAL ARTICLE

Transgenic expression of the forkhead box M1 transcription factor induces formation of lung tumors

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The forkhead box m1 (Foxm1 or Foxm1b) protein (previously called HFH-11B, Trident, Win or MPP2) is abundantly expressed in human non-small cell lung cancers where it transcriptionally induces expression of genes essential for proliferation of tumor cells. In this study, we used Rosa26-Foxm1 transgenic mice, in which the Rosa26 promoter drives ubiquitous expression of Foxm1 transgene, to identify new signaling pathways regulated by Foxm1. Lung tumors were induced in Rosa26-Foxm1 mice using the 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) lung tumor initiation/promotion protocol. Tumors from MCA/BHT-treated Rosa26-Foxm1 mice displayed a significant increase in the number, size and DNA replication compared to wild-type mice. Elevated tumor formation in Rosa26-Foxm1 transgenic lungs was associated with persistent pulmonary inflammation, macrophage infiltration and increased expression of cyclooxygenase-2 (Cox-2), Cdc25C phosphatase, cyclin E2, chemokine ligands CXCL5, CXCL1 and CCL3, cathepsins and matrix metalloprotease-12. Cell culture experiments with A549 human lung adenocarcinoma cells demonstrated that depletion of Foxm1 by either short interfering RNA transfection or treatment with Foxm1-inhibiting ARF 26-44 peptide significantly reduced Cox-2 expression. In co-transfection experiments, Foxm1 protein-induced Cox-2 promoter activity and directly bound to the −2566/−2580 bp region of human Cox-2 promoter.
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Keywords: winged helix DNA-binding domain; forkhead transcription factor; Foxm1; non-small cell lung cancer; lung epithelial cells; cellular proliferation

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Introduction

Lung cancer, which is the leading cause of death from cancer in the United States (Parkin *et al.*, 2005; Toloza *et al.*, 2006), is subdivided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Adenocarcinoma, the most common type of NSCLC, is frequently associated with gain of function mutations in the K-Ras oncogene, activation of the c-myc protein, as well as the loss of function mutations of tumor suppressor gene p53 (Giaccone, 1996; McCormick, 1999; Mitsuuchi and Testa, 2002; Sherr and McCormick, 2002). Activating mutations in K-Ras also occur in the majority of spontaneous and chemically induced mouse lung tumors (Malkinson, 1998). Inactivation of $p19^{ARF}$ and $p16^{INK}$ tumor suppressor genes due to promoter silencing is frequently associated with NSCLC (Sherr and McCormick, 2002).

The forkhead box (Fox) proteins are an extensive family of transcription factors, which share homology in the Winged Helix/*forkhead* DNA-binding domain (Clark *et al.*, 1993; Clevidence *et al.*, 1993). Expression of the Fox m1 (Foxm1) transcription factor is induced during cellular proliferation in a variety of different cell types and extinguished in terminally differentiated cells (Korver *et al.*, 1997; Ye *et al.*, 1997). Activation of the Ras–mitogen-activated protein kinase (MAPK) signaling pathway drives cell cycle progression by temporal expression of cyclin regulatory subunits, which activate their corresponding cyclin-dependent kinases (Cdk) through complex formation (McCormick, 1999; Sherr and McCormick, 2002). Cdk/cyclin complexes phosphorylate and activate a variety of the cell cycle regulatory proteins, including the Foxm1 protein (Major *et al.*, 2004). Activated MAPK (ERK) kinase has recently been shown to directly phosphorylate the Foxm1 protein, contributing to its transcriptional activation (Ma *et al.*, 2005). Foxm1 then directly stimulates the transcription of genes essential for progression into DNA replication and mitosis, including cyclin A2, cyclin B1, Cdc25B phosphatase, Aurora B kinase and Polo-like kinase 1 (Costa *et al.*, 2005). The Foxm1 protein also stimulates transcription of Skp2 and Cks1 genes, which are specificity subunits of the Skp1-Cullin-F-Box ubiquitin ligase complex that targets the Cdk inhibitors $p27^{kip1}$ and $p21^{cip1}$ for degradation during

the G_1/S transition (Wang *et al*., 2005). We previously demonstrated that *Foxm1* −/− mice exhibit embryonic lethality due to severe abnormalities in development of the heart, liver and lung (Krupczak-Hollis *et al*., 2004; Kim *et al*., 2005a). This is caused by a failure to complete mitosis, causing a significant reduction in the number of cells in these developing mouse organs (Krupczak-Hollis *et al*., 2004; Kim *et al*., 2005a).

Consistent with an important role of Foxm1 in cell cycle progression, increased expression of Foxm1 was found in human lung adenocarcinomas and squamous cell carcinomas (Kim *et al*., 2006), prostate adenocarcinomas (Kalin *et al*., 2006), basal cell carcinomas (Teh *et al*., 2002), intrahepatic cholangiocarcinomas (Obama *et al*., 2005), anaplastic astrocytomas and glioblastomas (van den Boom *et al*., 2003), infiltrating ductal breast carcinomas (Wonsey and Follettie, 2005), as well as in many other solid tumors (Pilarsky *et al*., 2004). Foxm1 is also overexpressed in hepatocellular carcinomas from patients who respond poorly to treatment (Lee *et al*., 2004). Depletion of Foxm1 in A549 cells caused diminished DNA replication and mitosis, reduced anchorage-independent growth of cell colonies on soft agar and reduced expression of cell cycle-promoting cyclin A2 and cyclin B1 (Kim *et al*., 2006). Partial deletion of mouse *Foxm1*-floxed targeted allele *in vivo* was sufficient to significantly reduce the number and size of lung adenomas induced by urethane (Kim *et al*., 2006). In this study, to identify new signaling pathways regulated by Foxm1, we used Rosa26-Foxm1 transgenic mice, in which the Rosa26 promoter drives ubiquitous expression of the Foxm1 transgene (Kalinichenko *et al*., 2003). We induced lung tumors in Rosa26-Foxm1 mice using the 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) lung tumor initiation/promotion protocol. Tumors from MCA/BHT-treated Rosa26-Foxm1 mice displayed a significant increase in number, size and DNA replication compared with (wild-type) WT mice. Increased lung tumorigenesis in Rosa26-Foxm1 transgenic mice was associated with persistent pulmonary inflammation, macrophage infiltration and increased expression of cyclooxygenase-2 (Cox-2). Diminished Cox-2 levels were observed in Foxm1-deficient lung tumors from Mx-Cre *Foxm1* fl/fl mice. Cell culture experiments with A549 cells demonstrated that Foxm1 induced Cox-2 promoter activity and directly bound to human Cox-2 promoter region.

Results

Ubiquitous expression of Foxm1 transgene increases the total number and size of lung tumors induced by MCA/BHT treatment

We previously demonstrated that Foxm1 protein is abundantly expressed in highly proliferative human NSCLC as well as in mouse lung tumors induced by urethane (Kim *et al*., 2006). To determine consequences of Foxm1 overexpression during formation of lung tumors, we used Rosa26-Foxm1 transgenic mice, which contain high levels of Foxm1 protein in all tissues (Kalinichenko *et al*., 2003). Lung tumors were induced in Rosa26-Foxm1 (experimental) and WT (control) FVB/N male mice by intraperitoneal (i.p.) injection of MCA, a polycyclic aromatic hydrocarbon found in tobacco smoke (Malkinson *et al*., 1997; Blaine *et al*., 2005). Starting 1 week after MCA injection, mice were subjected to weekly i.p. injections of BHT for 6 consecutive weeks to promote formation of lung tumors by causing chronic pulmonary inflammation and remodeling of the lung (Malkinson *et al*., 1997). On the basis of published studies (Blaine *et al*., 2005), we killed seven control WT mice and seven experimental Rosa26-Foxm1 transgenic mice at 12 and 24 weeks after initial MCA injection. Lungs were dissected and examined for tumors using a dissecting microscope. Total RNA was prepared from the left lung lobes and examined for levels of exogenous (transgenic human) Foxm1 and endogenous mouse Foxm1 mRNA using quantitative real-time reverse transcription (RT)–PCR (qRT–PCR). This method showed that the Foxm1 transgene is selectively expressed in Rosa26-Foxm1 lungs (Figure 1c), whereas low levels of endogenous Foxm1 were detected in both WT and Rosa26-Foxm1 lungs (Figure 1d).

Histological examination of Hematoxylin and eosin (H&E) stained lung sections revealed that all tumors in WT and Rosa26-Foxm1 transgenic mice displayed morphological characteristics of lung adenomas (Figure 2e, top panels). MCA/BHT-treated Rosa26-Foxm1 mice displayed a statistically significant increase in the total number of lung adenomas compared to WT mice (Figure 1a). Furthermore, by 24 weeks after MCA/BHT treatment, the diameter of lung tumors was significantly increased in Rosa26-Foxm1 mice (Figure 1b). These results suggest that transgenic overexpression of Foxm1 accelerates development and growth of lung tumors in MCA/BHT-treated mice.

DNA replication is increased in Rosa26-Foxm1 lungs following MCA/BHT treatment

We have previously demonstrated that a single administration of BHT to Rosa26-Foxm1 mice causes acute lung injury and elevated proliferation of epithelial, endothelial and smooth muscle cells during the period from 36 to 72 h following BHT injection (Kalinichenko *et al*., 2003). To determine whether increased tumor formation is associated with elevated proliferation in MCA/BHT-treated Rosa26-Foxm1 lungs, we compared DNA replication rates in lungs from WT and Rosa26-Foxm1 transgenic mice. DNA replication was determined by immunohistochemical detection of bromodeoxyuridine (BrdU) that had been administered in drinking water 4 days before killing the mice (Kalinichenko *et al*., 2004). Rosa26-Foxm1 tumors displayed a significant increase in the number of tumor cells undergoing DNA replication compared to WT tumors (Figure 2a, top panels; Figure 2c). Increased DNA replication rates were also observed in nontumor regions

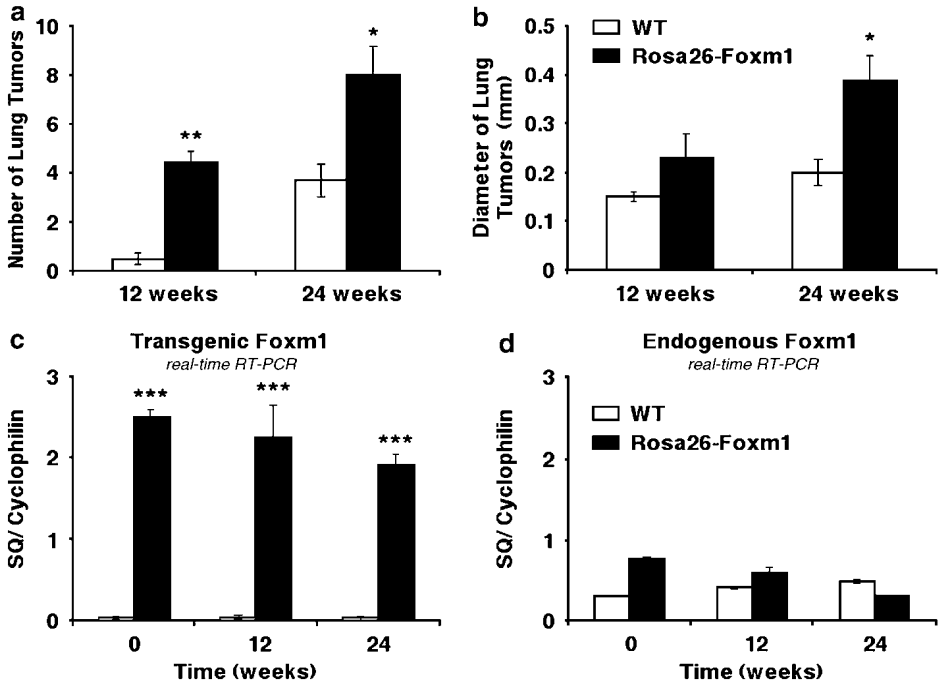


Figure 1 Increased expression of forkhead box m1 (Foxm1) in Rosa26-Foxm1 transgenic mice accelerates growth and development of lung tumors induced by 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) treatment. Rosa26-Foxm1 and wild-type (WT) mice were injected with a single dose of MCA, followed by six weekly injections of BHT to induce lung tumors as described in the 'Materials and methods'. Mice were killed at 12 weeks and 24 weeks after MCA injection and examined for lung tumors using a dissecting microscope. (**a**–**b**) Rosa26-Foxm1 transgenic mice displayed a significant increase in the total number of lung tumors (**a**) and tumor diameter (**b**). Mean number of tumors per lung (± s.d.) and mean diameter of lung tumor (± s.d.) were calculated using seven mouse lungs per each group. (**c**) Foxm1 transgene is selectively expressed in Rosa26-Foxm1 lungs. Expression of exogenous (transgenic) Foxm1 was examined in total lung RNA using quantitative real-time reverse transcription (RT)–PCR (qRT–PCR). Levels of cyclophilin mRNA were used to normalize expression levels of Foxm1 starting quantity SQ (SQ/cyclophilin). (**d**) Low levels of endogenous Foxm1 mRNA were detected in both WT and Rosa26-Foxm1 lungs. Expression of endogenous Foxm1 was not increased by MCA/BHT treatment. Asterisks (*) indicate statistically significant increases with *P*-values calculated by Student's *t*-test: $*P<0.05$, $**P<0.01$ and $***P<0.001$.

of Rosa26-Foxm1 lungs at 12 weeks after MCA/BHT treatment (Figure 2a and c), a result confirmed by immunostaining with antibodies against proliferation cell nuclear antigen (PCNA; Figure 2b). These results suggest that transgenic Foxm1 expression accelerates proliferation of lung tumor cells *in vivo*.

Rosa26-Foxm1 transgenic mice display persistent pulmonary inflammation following MCA/BHT treatment

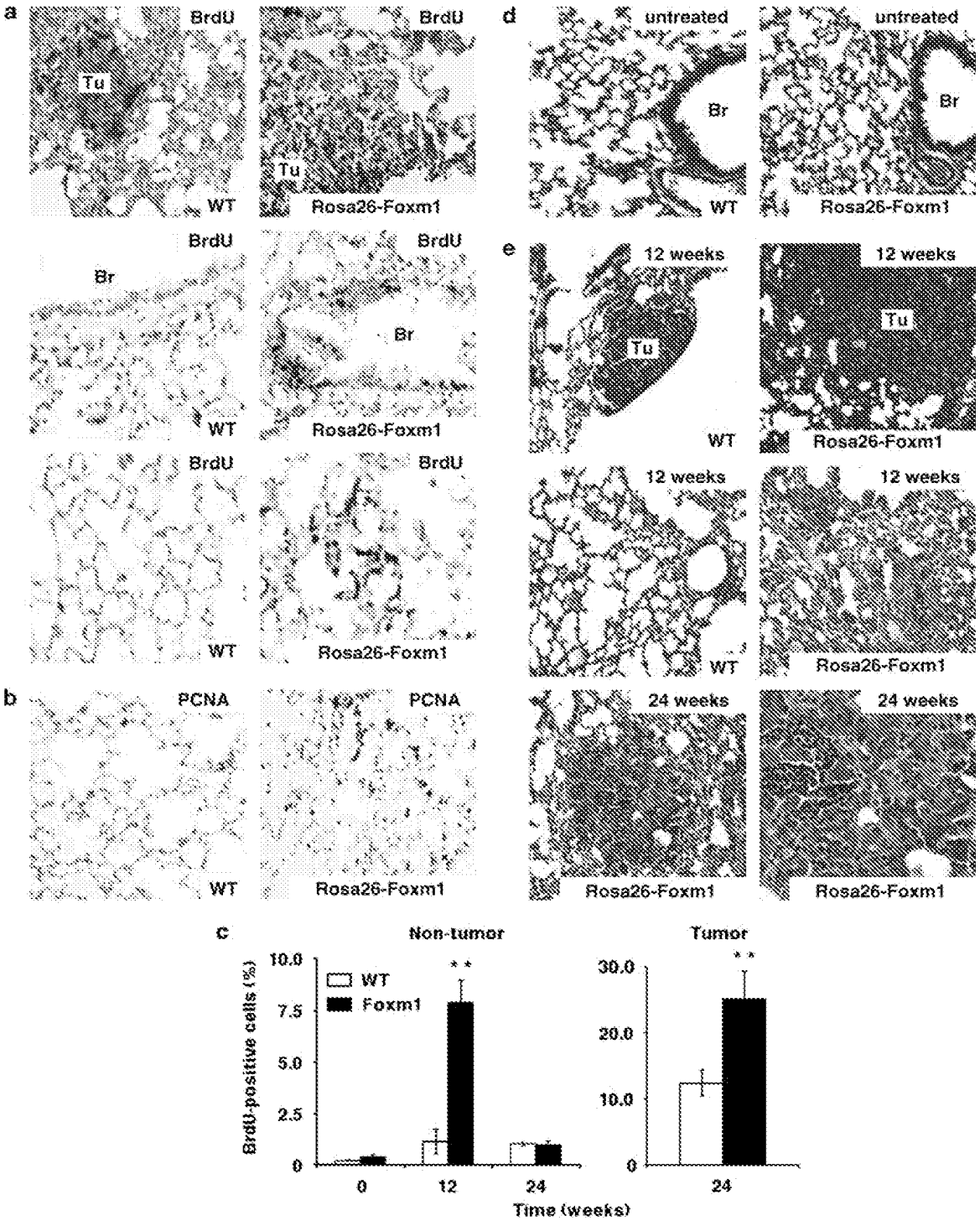
H&E staining demonstrated that untreated Rosa26-Foxm1 mice displayed normal lung structure, undistinguishable from WT lungs (Figure 2d). MCA/BHT treatment caused chronic lung inflammation in both Rosa26-Foxm1 and WT lungs. However, by 12 weeks after the initial treatment this inflammation was completely resolved in WT mice (Figure 2e, middle left panel), but not in Rosa26-Foxm1 mice. At 12 weeks after MCA treatment Rosa26-Foxm1 lungs continued to display pulmonary inflammation with thickening of alveolar septae and diffuse infiltration of hemosiderin-loaded macrophages (Figure 2e, middle right panel). The macrophage infiltration in Rosa26-Foxm1 lungs progressed to focal lung inflammation and fibrosis by 24 weeks after the initial MCA treatment (Figure 2e, bottom panels). Furthermore, lung tumors in Rosa26-Foxm1 mice were often observed in the focal inflammatory regions, suggesting that the increased inflammatory response in Rosa26-Foxm1 lungs contributed to lung carcinogenesis.

Gene expression profile in Rosa26-Foxm1 transgenic lungs after MCA/BHT treatment

In order to identify potential target genes regulated by Foxm1 during MCA/BHT-induced lung injury, we analysed mouse microarrays (Applied Biosystems, Foster City, CA, USA). These arrays were hybridized with cDNA probes synthesized from total lung RNA prepared from either WT or Rosa26-Foxm1 mice that were harvested at 12 weeks after the initiation of MCA/BHT treatment. This analysis identified pulmonary genes with altered expression levels in Rosa26-Foxm1 transgenic lungs compared to WT lungs (Table 2). Expression of 10 such Foxm1-responsive genes was further studied by qRT-PCR analysis using either untreated or MCA/BHT-treated WT or Rosa26-Foxm1 lungs (Figure 3). Consistent with elevated cellular proliferation in Rosa26-Foxm1 lungs (Figures 2a–c), we observed increased expression of the S-phase

promoting cyclin E2 and the Cdc25C phosphatase (Table 1; Figure 3), the latter of which activates Cdk1 during cellular progression into M-phase (Nilsson and Hoffmann, 2000).

Persistent pulmonary inflammation in Rosa26-Foxm1 lungs was associated with increased expression of Lipocalin-2, the CXC chemokine ligand 5 (CXCL5), CXCL1, and the CC chemokine ligand 3 (CCL3) (Figure 3), all of which stimulate signaling pathways essential for pulmonary inflammation and lung remodeling (Bratt, 2000; Kulbe *et al*., 2004). Interestingly, by 24 weeks after tumor initiation, expression levels of these genes were decreased in the Rosa26-Foxm1 lungs to approximately WT levels (Figure 3). Furthermore, lungs from MCA/BHT-treated Rosa26-Foxm1 mice displayed a significant increase in expression levels of



the matrix metalloprotease-12 (MMP-12), macrophage scavenger receptor-1 (MSR-1) and cathepsins D and K (Figure 3), confirming the increased number of macrophages in Rosa26-Foxm1 lungs (Figure 2e). Our results imply that transgenic Foxm1 overexpression stimulates expression of genes essential for lung inflammation and remodeling.

Rosa26-Foxm1 transgenic lungs exhibit elevated Cox-2 levels

Previous studies demonstrated that the expression of Cyclooxygenese-2 (Cox-2) is induced during pulmonary inflammation and lung carcinogenesis (Chun and Surh, 2004; Riedl *et al*., 2004). Since microarray analysis revealed increased Cox-2 levels in MCA/BHT-treated Rosa26-Foxm1 lungs (Table 2), we hypothesized that Foxm1 directly or indirectly regulates Cox-2 expression.

To investigate this possibility, we performed immunohistochemical staining with antibody specific for the Cox-2 protein. Although Cox-2 protein was undetectable in either untreated WT or untreated Rosa26-Foxm1 lungs, MCA/BHT-treatment induced Cox-2 expression in tumor cells and numerous types of nontumor cells in inflammatory regions, including alveolar type II and bronchial epithelial cells, macrophages, and neutrophils of both transgenic and control injured lungs (Figure 4a; data not shown). However, Cox-2 expression was significantly increased in MCA/BHT-treated Rosa26-Foxm1 lungs compared to WT lungs as demonstrated by increased Cox-2 protein immunostaining in Rosa26-Foxm1 lung tumors (Figure 4a) and qRT-PCR analysis of total lung RNA with primers specific to Cox-2 (Figure 4b). These results suggest that transgenic Foxm1 induces Cox-2 expression in MCA/BHT-treated lungs.

Decreased Cox-2 expression in Foxm1-deficient lung tumors from Mx-Cre Foxm1 fl/fl mice

We have previously demonstrated that treatment of Mx-Cre Foxm1 fl/fl mice with synthetic double-stranded (dsRNA; also known as PolyI:PolyC or PIPC) induced a 75% deletion of Foxm1-floxed allele in the lung during urethane-mediated lung carcinogenesis (Kim *et al*., 2006). This incomplete Foxm1 deletion resulted in formation of both Foxm1-negative and Foxm1-positive tumors in dsRNA-treated Mx-Cre Foxm1 fl/fl lungs (Kim *et al*., 2006). Therefore, we examined Cox-2 protein in these two types of Mx-Cre Foxm1 fl/fl lung tumors using immunohistochemical staining with Cox-2 antibody. Abundant Cox-2 expression was detected in Foxm1-positive lung tumors, whereas the Foxm1-negative tumors lacked detectable Cox-2 expression (Figure 4c). Furthermore, qRT-PCR analysis demonstrated that Cox-2 mRNA levels were significantly decreased in total lung RNA from dsRNA-treated Mx-Cre Foxm1 fl/fl mice when compared to either phosphate-buffered saline (PBS)-treated Mx-Cre Foxm1 fl/fl or dsRNA-treated Foxm1 fl/fl lungs (Figure 4d). These results suggest that Foxm1-deficiency is associated with reduced Cox-2 expression.

Foxm1 deficiency reduces Cox-2 expression in vitro

To determine whether Foxm1 directly regulates Cox-2 expression in A549 human lung adenocarcinoma cells, we transfected these cells with a short interfering RNA (siRNA) specific to the human Foxm1 mRNA (siFoxm1) or with a mutant control siFoxm1 duplex. Forty-eight hours after siRNA transfection, the cells were stimulated with lipopolysaccharide (LPS)/phorbol-12-myristate-13-acetate (PMA) for an additional 24 h to induce Cox-2 expression (Chang *et al*., 2005; Tong *et al*., 2006). Total RNA was prepared from the A549 cells and analysed for Foxm1 or Cox-2 levels by qRT-PCR. The siFoxm1 transfection efficiently reduced Foxm1 levels in A549 cells (Figure 5a) and caused a 50% decrease in the expression of Cox-2, whereas transfection of the mutant siFoxm1 did not influence expression levels of these genes (Figure 5a). Furthermore, a 50% decrease in Cox-2 expression was observed in A549 cells treated for 24 hours with a cell penetrating ARF 26-44 peptide (Figure 5b), a known peptide inhibitor of Foxm1 transcriptional activity (Kalinichenko *et al*., 2004). The ARF 26-44 peptide was fused to nine N-terminal D-Arg residues for efficient transduction into the cells (Kalinichenko *et al*., 2004).

Figure 2 Rosa26-Foxm1 lungs display increased cell proliferation and persistent pulmonary inflammation following 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) treatment. (**a**) DNA replication is increased in MCA/BHT-treated Rosa26-Foxm1 lungs. Paraffin sections were prepared from either wild-type (WT) or Rosa26-Foxm1 transgenic lungs at 12 weeks after initial MCA injection. DNA replication was determined by immunohistochemical detection of bromodeoxyuridine (BrdU) that had been administered in drinking water 4 days before killing the mice. MCA/BHT-treated Rosa26-Foxm1 mice displayed increased numbers of BrdU-positive cells in lung tumors (Tu, shown with arrowheads) (top right), bronchial (Br) epithelial cells (middle right) and alveolar epithelial cells (bottom right) when compared to WT mice (left panels). (**b**) Increased expression of the proliferation cell nuclear antigen (PCNA) in MCA/BHT-treated Rosa26-Foxm1 transgenic lungs. (**c**) Increased numbers of BrdU-positive cells in Rosa26-Foxm1 lungs. BrdU-positive cells were counted in ten random microscope fields from either tumor or nontumor regions of Rosa26-Foxm1 and WT lungs (mean ± s.d.). Asterisks (**) indicate statistically significant *P*-values $\leqslant 0.01$. (**d**) Hematoxylin and eosin (H&E) staining of lung sections from untreated WT and Rosa26-Foxm1 transgenic mice (**e**) H&E staining of MCA/BHT-treated WT and Rosa26-Foxm1 lungs. All tumors in WT and Rosa26-Foxm1 transgenic lungs displayed morphological characteristics of lung adenomas (top panels). Pulmonary inflammation in WT lungs was completely resolved by 12 weeks after tumor initiation (middle left). Rosa26-Foxm1 transgenic mice displayed persistent pulmonary inflammation with thickening of alveolar septae and diffuse infiltration of hemosiderin-loaded macrophages (middle right). By 24 weeks after tumor initiation, Rosa26-Foxm1 lungs displayed pulmonary granuloma containing macrophages (bottom left). Higher magnification of the previous section shows granular brown pigment hemosiderin within macrophages (bottom right). Magnification: all (**a**) panels, and bottom right panel (**e**), ×200; (**b**–**d**) panels and remaining (**e**) panels, ×100.

Table 1 PCR oligonucleotides

Gene	*Forward primer (5′–3′)*	*Reverse primer (5′–3′)*
mCox-2	TCAGTAGGTTTTTGCTGTGAGG	GTTCAATGGGCTGGAAGACA
hCox-2	GGTCTGGTGCCTGGTCTGATGATG	GTCCTTTCAAGGAGAATGGTGC
mCdc25C	TTCTGAGGAAGCCTGTTGTT	AAATCTCTGTAGCCCCCTTTG
mLipocalin-2	CCACCACGGACTACAACCA	CCACACTCACCACCCATTC
mCXCL5	TGAAGAAGGTAAGAAGCAAGGAA	GGCTTAAAATAATGGATGTGATG
mCXCL1	ATGTGTGGGAGGCTGTGTTT	AGCGAGACGAGACCAGGAG
mCCL3	CTGCCTGCTGCTTCTCCTAC	GTTCCTCGCTGCCTCCAA
mMMP-12	CCTGACACGGGAAAAACATC	CACCAGCATAAAAATAGCAGAAA
mCathepsin K	TGGCTCGGAATAAGAACAAC	CACTGGAAGCACCAACGA
mMSR1	CCGAGGAAAAGAAAGATGGA	CACAAGAAAAGAGTCACCCACA
mCathepsin D	TGCTGAGATGGATTGTCTTG	CAGAGTTGGAGGGGCAGTAG
hCox-2 (no. 1)[a]	CAGTTCAGGTTTTCCCTACCCAGG	AGAAGTGAGGCGACAGGTCATAAC
hCox-2 (no. 2)[a]	ATCGCCGCTTCCTTTGTCCATC	TCACCCCCTCCTTGTTTCTTGG

Abbreviations: ChIP, chromatin immunoprecipitation; qRT–PCR, Quantitative real-time–PCR. [a]Used for ChIP (all other primer sets were used for qRT-PCR).

Cox-2 levels remained unchanged in A549 cells when treated with a truncated form of the ARF 37–44 peptide (Figure 5b) which lacked the amino acids 26–36 required to interact with the Foxm1 protein (Kalinichenko *et al.*, 2004; Gusarova *et al.*, 2007). These results demonstrate that inhibition of Foxm1 by either siRNA transfection or the ARF 26-44 peptide treatment reduces Cox-2 expression in A549 human lung adenocarcinoma cells.

To determine whether Foxm1 directly stimulates Cox2 promoter activity, co-transfection experiments were performed in A549 cells using a cytomegalovirus (CMV)-Foxm1 expression vector and luciferase (LUC) reporter driven by the −3.2 kb mouse Cox-2 promoter region. Co-transfection of the CMV-Foxm1 expression vector increased expression of the −3.2 kb Cox-2-LUC reporter plasmid compared to CMV-empty vector (Figure 5c).

We next used chromatin immunoprecipitation (ChIP) assays to determine whether Foxm1 protein directly binds to the human Cox-2 promoter region in the context of endogenous DNA. The cross-linked and sonicated chromatin from untransfected A549 cells or A549 cells transfected with either siFoxm1 or mutant siFoxm1 was immunoprecipitated (IP) with specific anti-Foxm1 antibodies or with immunoglobulin G control antibodies. Binding of Cox-2 promoter DNA associated with the IP chromatin was determined by the real-time PCR with primers specific to potential Foxm1-binding sites in human Cox-2 promoter (Figure 5d). Although Foxm1 did not recognize the −1473/−1487 Cox-2 promoter region, it did specifically bind the −2566/−2580 bp region as demonstrated by the ability of siFoxm1 to reduce binding of Foxm1 protein to the Cox-2 promoter region (Figure 5d).

Discussion

Foxm1 transcription factor regulates expression of genes essential for DNA replication and mitosis during a variety of biological processes including embryonic development, organ injury and cancer formation (Kalinichenko *et al.*, 2003; Krupczak-Hollis *et al.*, 2004; Kim *et al.*, 2005a). Consistent with the important role of Foxm1 in cell cycle progression, elevated Foxm1 levels have been found in a variety of tumor cell lines (Korver *et al.*, 1997; Ye *et al.*, 1997) as well as numerous types of human tumors (Teh *et al.*, 2002; van den Boom *et al.*, 2003; Lee *et al.*, 2004; Obama *et al.*, 2005; Wonsey and Follettie, 2005; Kalin *et al.*, 2006). Our recent studies demonstrated that Foxm1 protein is abundantly expressed in highly proliferative human NSCLC as well as in mouse lung tumors induced by urethane (Kim *et al.*, 2006). A 75% deletion of *Foxm1* fl/fl-targeted alleles in Mx-Cre *Foxm1* fl/fl mice was sufficient to reduce the number and size of lung adenomas following urethane treatment (Kim *et al.*, 2006).

The aim of the current study was to identify new signaling pathways regulated by Foxm1 during formation of NSCLC. We used Rosa26-Foxm1 transgenic mice, in which the Rosa26 promoter drives ubiquitous expression of the Foxm1 transgene (Kalinichenko *et al.*, 2003). This transgenic mouse model allowed us for the first time to evaluate the consequences of Foxm1 overexpression during lung tumorigenesis induced by MCA/BHT treatment. MCA/BHT-treated Rosa26-Foxm1 mice displayed a significant increase in the proliferation of lung tumor cells as well as in the number and size of lung adenomas, further confirming the important role of Foxm1 in proliferation of tumor cells during development and progression of NSCLC. MCA/BHT-treated Rosa26-Foxm1 lungs also showed increased expression of the M-phase promoting Cdc25C phosphatase, which is consistent with reduced expression of Cdc25C in developing lungs from *Foxm1* −/− embryos (Kim *et al.*, 2005a). Interestingly, untreated Rosa26-Foxm1 lungs exhibited WT levels of Cdc25C and other Foxm1 target genes despite increased expression of the transgenic Foxm1 mRNA. One possible explanation for this discrepancy is that Foxm1 transgenic protein is transcriptionally inactive in quiescent lungs. It was previously demonstrated (Ye *et al.*, 1999) that in quiescent cells transgenic Foxm1 protein localizes in cytoplasm and needs an activation signal (injury-induced proliferative stimuli) to be translocated

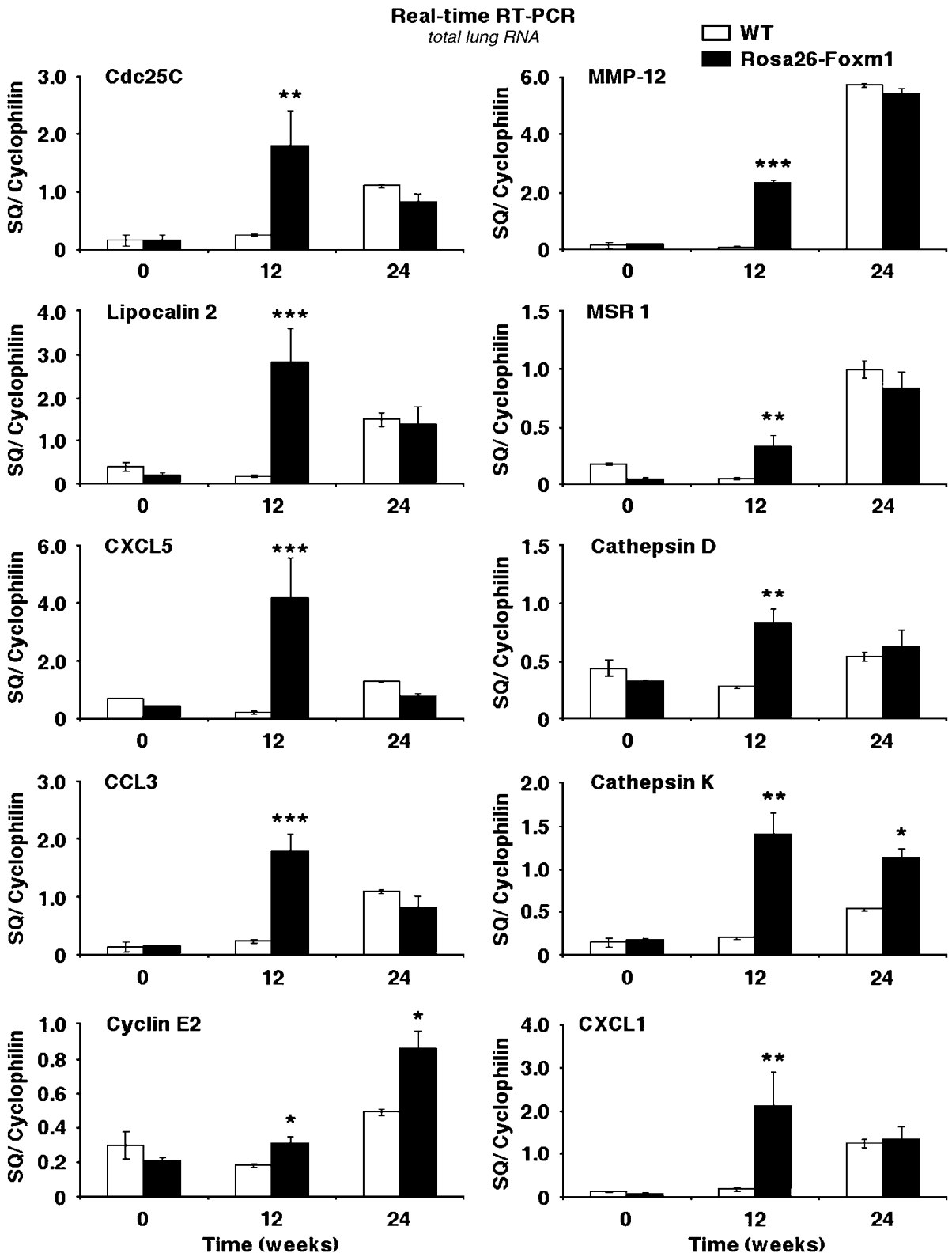


Figure 3 Gene expression profile in Rosa26-Foxm1 transgenic lungs after 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) treatment. Total RNA was prepared from left lung lobes of untreated or MCA/BHT-treated wild-type (WT) and Rosa26-Foxm1 mice. Quantitative real-time reverse transcription (RT)–PCR (qRT–PCR) was performed with primers specific to CDC25C, cyclin E2, lipocalin 2, chemokine ligands CXCL5, CCL3 and CXCL1, the matrix metalloprotease-12 (MMP-12), macrophage scavenger receptor-1 (MSR-1), cathepsins D and K, and cyclophilin. Each individual sample was normalized to its corresponding cyclophilin level (SQ/cyclophilin). Asterisks (*) indicate statistically significant differences with *P*-values calculated by Student's *t*-test: $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Table 2 Gene expression changes in Rosa26-Foxm1 transgenic lungs after MCA/BHT treatment

Gene	*ID number*	*Fold change*[a]
Inflammation/chemotaxis		
Chemokine {C-X-C motif} ligand 5 (CXCL 5)	mCG1701.2	21.6
Lipocalin 2	mCG17450.2	18.7
Chemokine {C-C motif} ligand 7 (CCL 7)	mCG52384.1	13.7
Chemokine {C-X-C motif} ligand 1 (CXCL 1)	mCG1708.2	12.1
Chemokine {C-C motif} ligand 3 (CCL 3)	mCG11624	7.1
Chemokine {C-C motif} ligand 2 (CCL 2)	mCG8184.2	5.0
Chemokine {C-X-C motif}ligand 2 (CXCL 2)	mCG1710	3.8
Chemokine {C-C motif} ligand 6 (CCL 6)	mCG11623.1	3.7
Chemokine {C-C motif} ligand 9 (CCL 9)	mCG19020.1	2.8
Prostaglandin 1 receptor (IP)	mCG16538.2	2.1
Interleukin 10	mCG2645.1	0.4
Extracellular matrix remodeling		
Matrix metalloproteinase-12 (MMP-12)	mCG15953.2	12.7
MMP-13	mCG15946.2	4.9
Laminin, $\alpha 2$	mCG7304.2	3.5
MMP-1α	mCG128874.1	2.6
MMP-7	mCG9895.1	2.1
MMP-19	mCG18673.2	2.0
Cyclooxygenase-2 (Cox-2)	mCG29254	2.0
Procollagen, type IV, $\alpha 3$	mCG118161.1	0.4
MMP-28 (epilysin)	mCG11680.3	0.3
Procollagen, type VIII, $\alpha 1$	mCG129488.2	0.3
Macrophage functions		
Cathepsin K	mCG16733.3	5.7
Macrophage scavenger receptor-1 (MSR-1)	mCG18344.2	3.6
Cathepsin D	mCG11067.2	2.9
Colony-stimulating factor 2 (granulocyte-macrophage)	mCG13784.2	2.9
Cathepsin Z	mCG9962.3	2.3
Cathepsin L	mCG14471	2.0
Proliferation		
Cyclin A2	mCG19561.1	3.9
Cyclin E2	mCG5098.2	2.7
Cell division cycle 25 C (Cdc25C)	mCG123910.1	2.6
Cyclin M4	mCG127201.1	2.5
Proliferin 2	mCG113135.1	2.4
Growth arrest-specific 2	mCG19421.2	0.4
Suppression of tumorigenicity 5	mCG120215.1	0.02

[a]Total RNA was prepared from lungs of WT ($n=3$) or Rosa26-Foxm1 transgenic mice ($n=3$) taken 12 weeks after MCA/BHT treatment. Fold change in gene expression is presented as Rosa26-Foxm1 to WT signal ratio.

into nucleus and to stimulate transcription of its target genes.

Foxm1 expression transiently increases during acute lung injury and remains elevated until lung repair is completed (Kalinichenko *et al*., 2001b; Zhao *et al*., 2006). However, no significant differences in acute pulmonary inflammation were reported in previous studies for either BHT-treated Rosa26-Foxm1 mice (Kalinichenko *et al*., 2003) or LPS-treated Tie2-Cre *Foxm1* fl/fl mice (Zhao *et al*., 2006). In this study, we demonstrated for the first time that Foxm1 plays a role in chronic lung inflammation, a known predisposing factor for tumor development. Although pulmonary inflammation was completely resolved by 12 weeks after initiation of MCA/BHT treatment in WT mice, Rosa26-Foxm1 mice displayed persistent pulmonary inflammation, macrophage infiltration, and increased expression of genes essential for pulmonary inflammation and remodeling in response to lung injury (Bratt, 2000; Wolters and Chapman, 2000; Chun and Surh, 2004; Kulbe *et al*., 2004; Riedl *et al*., 2004; Acuff *et al*., 2006). Therefore, the observed changes in gene expression probably represent the increased inflammatory response in MCA/BHT-treated Rosa26-Foxm1 lungs compared to WT lungs. The absence of inflammation in untreated Rosa26-Foxm1lungs (Figure 2d), indicates that MCA/BHT treatment induced this inflammatory response. Our results suggest that Foxm1 overexpression induces an enhanced inflammatory response in chronically injured lungs. Alternatively, Foxm1 overexpression may inhibit the resolution of lung inflammation caused by chronic MCA/BHT treatment.

Sustained inflammation and increased tumorigenesis in Rosa26-Foxm1 lungs was associated with increased expression of Cox-2, a well-known marker of inflammation and a critical player in carcinogenesis

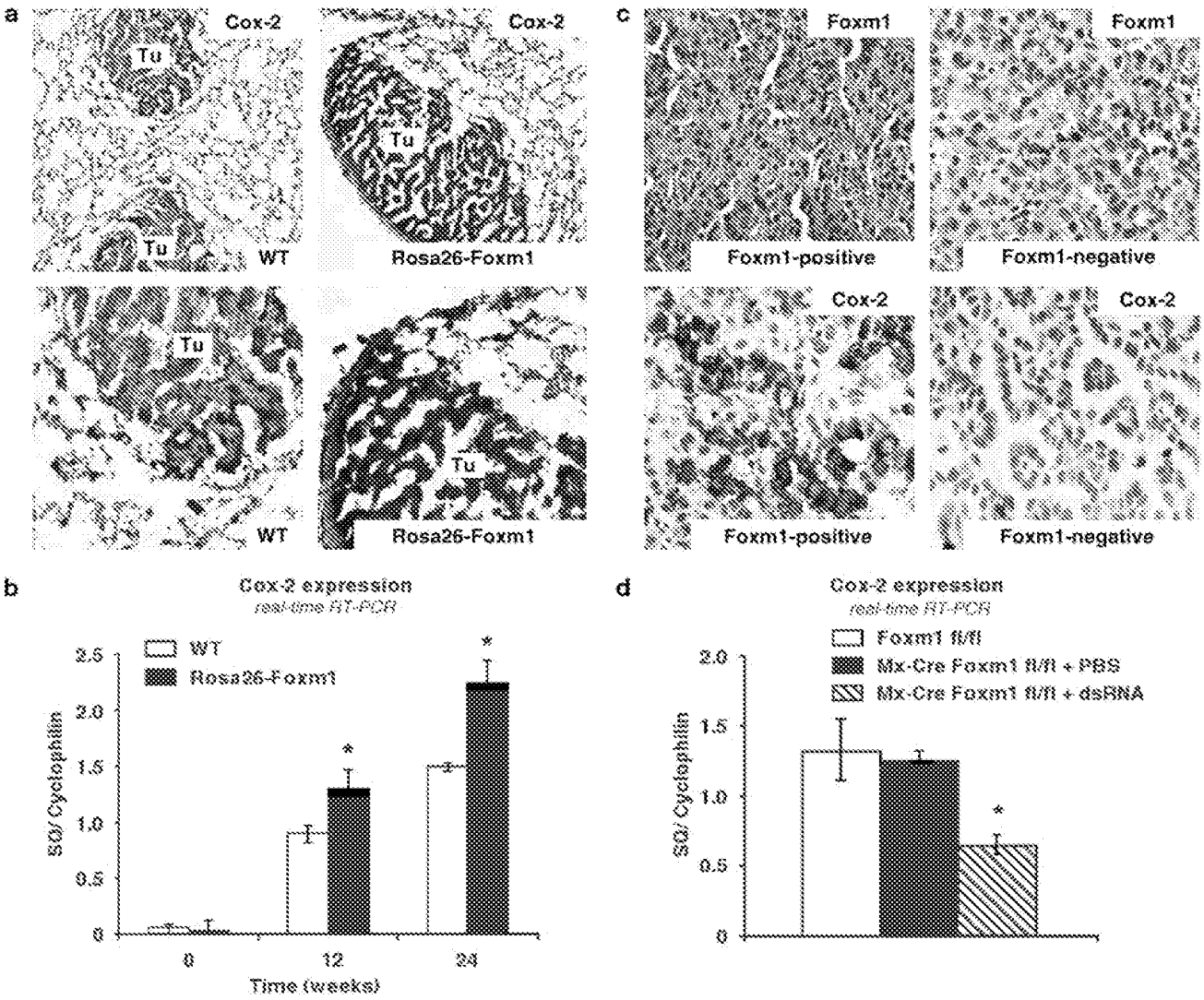


Figure 4 Forkhead box m1 (Foxm1) expression positively correlates with cyclooxygenase-2 (Cox-2) levels in lung tumors. (**a**–**b**) Rosa26-Foxm1 transgenic lungs exhibit elevated Cox-2 levels. Lung paraffin sections from wild-type (WT) and Rosa26-FoxM1 TG mice treated with 3-methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) for 24 weeks were immunochemically stained with antibodies specific to Cox-2 (dark purple in **a**). Slides were counterstained with nuclear fast red (red in **a**). Cox-2 protein levels were increased in lung tumors (Tu) from Rosa26-Foxm1 mice (right panes in **a**) compared to WT lung tumors (left panels in **a**). Quantitative real-time reverse transcription (RT)–PCR (qRT–PCR) analysis with total lung RNA demonstrated that the Rosa26-Foxm1 lungs displayed statistically significant increases in Cox-2 mRNA levels at both 12 and 24 weeks after tumor initiation (**b**). Levels of cyclophilin mRNA were used to normalize expression levels of Cox-2 in the lung. Asterisks (*) indicate statistically significant *P*-values <0.05. (**c**–**d**) Decreased Cox-2 expression in Foxm1-deficient Mx-Cre *Foxm1* fl/fl lungs. Mx-Cre *Foxm1* fl/fl mice and control *Foxm1* fl/fl mice were intraperitoneally (i.p.) injected three times with 250 μl of synthetic double-stranded RNA (dsRNA; also known as PolyI:PolyC or PIPC; 1.25 mg ml^{-1} in phosphate-buffered saline (PBS)) to induce the deletion of *Foxm1* fl/fl-targeted allele. After 1 week mice were i.p. injected with 1 g per kg of urethane once a week for 10 consecutive weeks. Mice were killed at 28 weeks following the initial urethane injection. The treatment of Mx-Cre *Foxm1* fl/fl mice with synthetic dsRNA induced a 75% deletion of Foxm1-floxed allele in the lung during urethane-mediated lung carcinogenesis, which resulted in formation of both Foxm1-negative and Foxm1-positive tumors in Mx-Cre *Foxm* fl/fl lungs. Adjacent paraffin sections from either Foxm1-positive (left panels in **c**) or Foxm1-negative (right panels in **c**) lung tumors from Mx-Cre *Foxm1* fl/fl mice were stained with Foxm1 antibody (top panels in **c**) or Cox-2 antibody (bottom panels in **c**). Foxm1-stained sections were counterstained with hematoxylin (blue), whereas Cox-2-stained sections were counterstained with nuclear fast red (red). qRT–PCR analysis demonstrated that Cox-2 levels are significantly decreased in total lung RNA prepared from dsRNA-treated Mx-Cre *Foxm1* fl/fl mice when compared to either control PBS-treated Mx-Cre *Foxm1* fl/fl lungs or control dsRNA-treated *Foxm1* fl/fl lungs. Three mouse lungs were used for each group. Cyclophilin mRNA levels were used to normalize expression levels of Cox-2 in the lung. A *P*-value <0.05 is shown with asterisk (*). Magnification: top (**a**) panels, $\times 50$; bottom (**a**) panels, $\times 200$; (**c**) panels, $\times 400$.

(Chun and Surh, 2004; Riedl *et al*., 2004). Elevated Cox-2 expression has been found in a variety of human cancers including NSCLC (Chun and Surh, 2004; Riedl *et al*., 2004). Our results are consistent with the possibility that Foxm1-dependent Cox-2 induction contributes to sustained inflammatory response and elevated tumorigenesis in MCA/BHT-treated Rosa26-Foxm1 lungs.

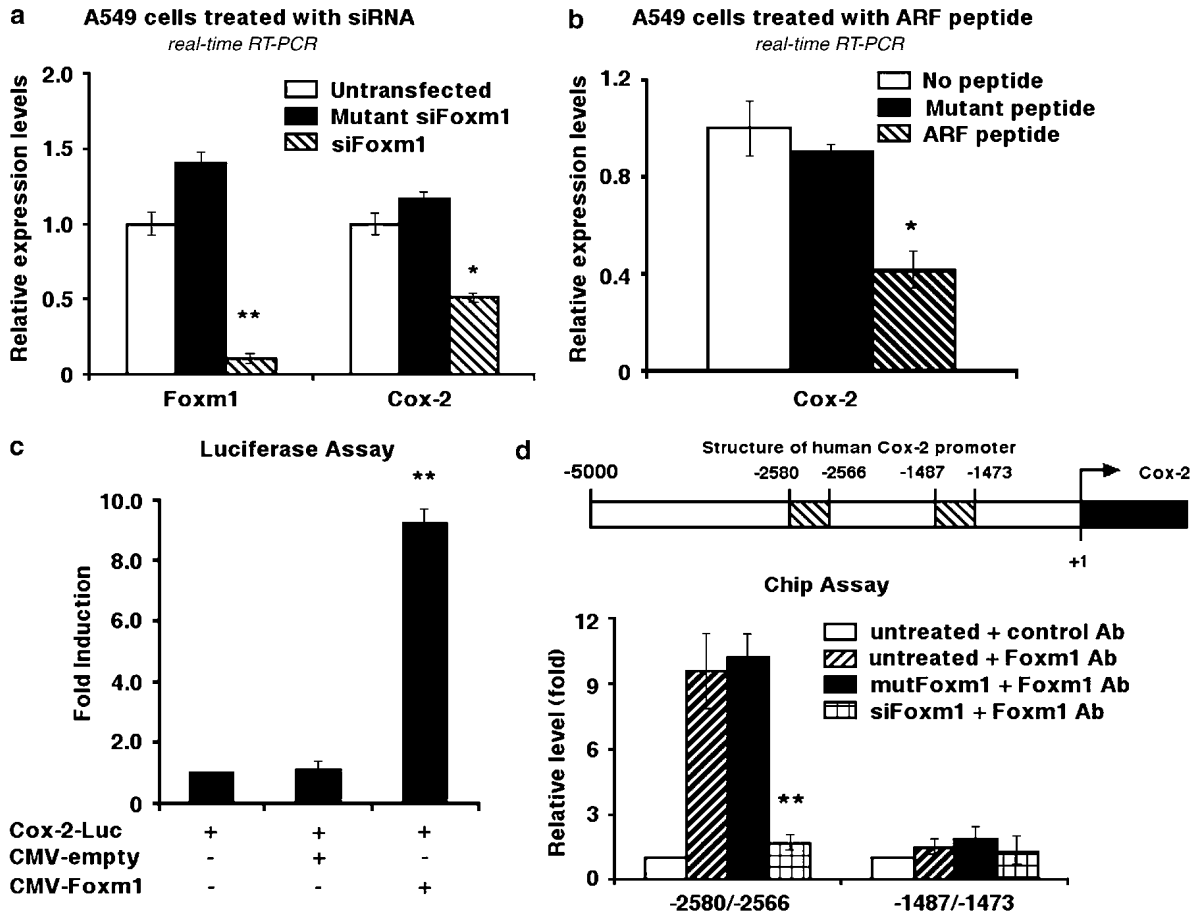


Figure 5 Forkhead box m1 (Foxm1) induces cyclooxygenase-2 (Cox-2) expression *in vitro*. (**a**) Foxm1 depletion in A549 cells reduces Cox-2 expression. A549 human lung adenocarcinoma cells were transfected with short interfering RNA (siRNA) duplex specific to the human Foxm1 cDNA (siFoxm1) or with mutant control siFoxm1 duplex. Forty-eight hours after siRNA transfection, the cells were stimulated with LPS/PMA for an additional 24 h to induce the Cox-2 expression. Total RNA was analysed for Foxm1 and Cox-2 mRNA levels by the real-time reverse transcription (RT)–PCR with primers specific to human Foxm1 and Cox-2, respectively. Cyclophilin mRNA levels were used to normalize expression levels of Foxm1 and Cox-2. (**b**) Foxm1 depletion by ARF 26–44 peptide causes a significant decrease in Cox-2 expression. A549 cells were treated with either ARF 26–44 peptide or control ARF 37–44 peptide in the presence of LPS/PMA. Total RNA was analysed for Cox-2 and cyclophilin levels by the real-time RT-PCR. (**c**) Foxm1 induces Cox-2 promoter activity in co-transfection assays. We transiently transfected U2OS cells with cytomegalovirus (CMV)-Foxm1 expression vector and the −3.2 kb mouse Cox-2 promoter-luciferase (LUC) reporter plasmid. Cells were harvested at 24 h after transfection and processed for dual luciferase assays to determine luciferase activity. (**d**) Schematic drawing of the −5 kb promoter region of the human Cox-2 gene (top panel) with two potential Foxm1 DNA-binding sites: −1487/−1473 and −2580/−2566. Foxm1 directly binds to −2580/−2566 region of the human Cox-2 promoter (bottom panel). Cross-linked and sonicated chromatin from untransfected A549 cells or A549 cells transfected with either siFoxm1 or mutant siFoxm1 was immunoprecipitated (IP) with antibodies specific to either Foxm1 antibody or rabbit serum (control Ab). Binding of Cox-2 promoter DNA associated with the IP chromatin was determined by real-time PCR with primers specific to potential Foxm1-binding sites in human Cox-2 promoter. Foxm1-depleted A549 cells showed diminished binding of Foxm1 to the endogenous human promoter regions of the Cox-2 gene. Asterisks (*) indicate statistically significant changes, with *P*-values calculated by the Student's *t*-test as follows: $*P<0.05$; $**P<0.01$.

We previously synthesized a cell penetrating ARF 26-44 peptide fused to nine N-terminal D-Arg residues (Kalinichenko *et al.*, 2004; Gusarova *et al.*, 2007), which is efficiently transduced into cells, and inhibits Foxm1 transcriptional activity by direct binding to the Foxm1 protein (Kalinichenko *et al.*, 2004). In this study, we demonstrated that depletion of Foxm1 by either ARF 26–44 peptide or siRNA transfection causes reduced Cox-2 expression in A549 human lung adenocarcinoma cells, suggesting that Foxm1 induces Cox-2 expression in lung tumor cells. Because Foxm1 protein stimulates Cox-2 promoter activity and directly binds to Cox-2 promoter region, our data suggest that Foxm1 is a transcriptional activator of the Cox-2 gene. These results are consistent with decreased Cox-2 expression in total lung RNA from Foxm1-deficient Mx-Cre *Foxm1* fl/fl mice.

The Rosa26-Foxm1 transgene is overexpressed in all cell types of the lung, and a single BHT injection caused increased cellular proliferation in epithelial, endothelial and smooth muscle cells in injured Rosa26-Foxm1 lungs (Kalinichenko *et al.*, 2003). Therefore, increased tumor formation in Rosa26-Foxm1 transgenic mice can be explained by direct Foxm1 effect on proliferation of

epithelial-derived tumor cells, or by effects of Foxm1 on tumor angiogenesis. This is consistent with previous reports demonstrating that Foxm1-depletion by siRNA transfection inhibits proliferation of both human adenocarcinoma A549 cells (Kim *et al*., 2006) and human microvascular endothelial HMEC-1 cells (Zhao *et al*., 2006). However, we were unable to detect significant differences in either the morphological appearance of pulmonary endothelial cells or the expression levels of endothelial-specific Pecam-1 protein in WT and Rosa26-Foxm1 lungs.

In summary, MCA/BHT-treated Rosa26-Foxm1 mice displayed persistent pulmonary inflammation and increased number of lung tumors. MCA/BHT-treated Rosa26-Foxm1 lungs also expressed increased levels of Cox-2, whereas Cox-2 levels were diminished in Foxm1-deficient lung tumors from Mx-Cre *Foxm1* fl/fl mice. Foxm1 induced Cox-2 expression in cultured A549 cells. Foxm1 stimulates Cox2 promoter activity in co-transfection experiments. Foxm1 protein directly binds to Cox-2 promoter in context of endogenous chromatin.

Materials and methods

Transgenic mice and tumor induction protocol
We previously described development of Rosa26-Foxm1 FVB/N transgenic mice, in which the Rosa26 promoter drives ubiquitous expression of the human Foxm1b cDNA in all cell types (Kalinichenko *et al*., 2003). Rosa26-Foxm1 mice and WT FVB/N mice were injected i.p. with a single dose of MCA (15 $\mu g\,g^{-1}$ mouse weight), followed by six weekly i.p. injections of BHT (the first dose was 150 $\mu g\,g^{-1}$ mouse weight, and subsequent doses were 200 $\mu g\,g^{-1}$ mouse weight) as described (Blaine *et al*., 2005). Seven mice from each group were killed at 12 weeks and 24 weeks after MCA injection and examined for lung tumors using a dissecting microscope. Lung tissues were either used to prepare total RNA or fixed, paraffin embedded, sectioned, and then used for histological staining with H&E or for immunohistochemistry.

BrdU labeling and immunohistochemical staining
In order to monitor the proliferation of tumor cells, mice were placed on drinking water containing 1 $mg\,ml^{-1}$ of 5-bromo-2′-deoxyuridine (BrdU) for four days and then they were killed (Kalinichenko *et al*., 2004). Cell proliferation rates were detected by immunohistochemical staining of mouse lung paraffin sections using mouse monoclonal antibodies specific for BrdU (1:100; clone Bu 20A; Dako, Carpinteria, CA, USA) or PCNA (1:1000; clone PC-10; Roche Diagnostics, Indianapolis, IN, USA). Antibody-antigen complexes were detected by anti-mouse antibody conjugated with alkaline phosphatase and BCIP/NBT substrate (all from Vector Lab) as described (Kalinichenko *et al*., 2001a, 2004; Kim *et al*., 2005a). In each mouse lung, we counted the number of BrdU-positive cells per 1000 cells in ten distinct 400 × microscope fields.

Lung paraffin sections were also stained with goat polyclonal Cox-2 antibody (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or rabbit polyclonal Foxm1 antibody (1:200; (Kalinichenko *et al*., 2004)) followed by biotinylated secondary antibodies and avidin conjugated with either horseradish peroxidase (HRP; for Foxm1 detection) or alkaline phosphatase (AP, for Cox-2 detection). The 3,3′-diaminobenzidine (DAB, Vector Lab, Burlingame, CA, USA) was used as HRP substrate, whereas BCIP/NBT (Vector Lab) was used as AP substrate as previously described (Kim *et al*., 2005a, b).

Analysis of expression arrays
The Mouse Genome Survey Microarray (P/N 4345065; Applied Biosystems, Foster City, CA, USA) contains 32 996 probes, representing 32 381 genes that target 44 498 transcripts. A digoxigenin-UTP-labeled probe was generated and amplified from 2 μg of total RNA prepared from the left lung lobes (containing nontumor and tumor regions) of either WT or Rosa26-Foxm1 mice treated with MCA/BHT for 12 weeks. To avoid individual variations in gene expression, each RNA sample represented a mixture of equal RNA amounts from three distinct mice. Array hybridization and analysis were performed in the Functional Genomic Facility of the University of Chicago. Expression signals were normalized to expression levels of cyclophilin and β-actin using the 1700 Chemiluminescent Microarray Analyzer software v 1.1 (Applied Biosystems). In Table 2, we summarize our focus on characterization of 34 genes whose expression levels were altered more then twofold in MCA/BHT-treated Rosa26-Foxm1 lungs compared to WT lungs. To verify expression levels of these genes we performed qRT-PCR analysis.

Quantitative real-time RT–PCR
To remove contaminating genomic DNA, total lung RNA was digested with RNAse-free DNAse I and then purified using the RNeasy Micro Kit (catalog no. 74004; Qiagen). We used the cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) containing both oligo-dT and random hexamer primers to synthesize cDNA from 10 μg of total RNA as described (Kim *et al*., 2006). qRT-PCR primers for mouse and human Foxm1 and cyclophilin were previously described (Kim *et al*., 2006). qRT-PCR primers for other mouse and human genes are listed in Table 1. Expression levels were normalized to cyclophilin mRNA levels and presented as means ± s.d.

Transfection of A549 cells with siRNA duplexes
We transfected A549 cells with 100 $nM\,l^{-1}$ of either siFoxm1 or mutant control siFoxm1 using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in serum-free tissue culture medium as previously described (Wang *et al*., 2005; Kim *et al*., 2006). Forty-eight hours after transfection, the A549 cells were stimulated with PMA (100 nM) and LPS (2.5 $\mu g\,ml^{-1}$) in the presence of siRNA to induce Cox-2 expression as described (Chang *et al*., 2005; Tong *et al*., 2006). After 24 hours of LPS/PMA treatment, these cells were used to prepare total RNA using RNA-STAT-60 (Tel-Test "B", Inc., Friendswood, TX, USA).

ARF 26–44 peptide treatment
Genemed Synthesis Inc. (South San Francisco, CA, USA) synthesized a $(D\text{-}Arg)_9$-ARF 26–44 peptide (rrrrrrrrrKFV RSRRPRTASCALAFVN) containing nine D-Arg residues at the N terminus, which has been demonstrated to enhance cellular uptake of polypeptides (Wender *et al*., 2000). We previously demonstrated that the $(D\text{-}Arg)_9$-ARF 26–44 peptide directly binds to endogenous Foxm1 protein, targets it to the nucleolus and inhibits Foxm1 transcriptional activity (Kalinichenko *et al*., 2004; Gusarova *et al*., 2007). As a control, we used mutant $(D\text{-}Arg)_9$-ARF 37–44 peptide, which lacked the amino acids 26–37 required to interact with the Foxm1 protein (Kalinichenko *et al*., 2004; Gusarova *et al*., 2007). A549 cells were treated with 10 μM of either ARF 26-44 peptide or mutant ARF 37–44 peptide for 6 h and then stimulated with

PMA (100 nM) and LPS (2.5 mg ml^{-1}) for an additional 18 h in the presence of peptide.

Co-transfection studies and ChIP assays

We transfected A549 cells with −3.2 kb mouse Cox-2 promoter—LUC construct (catalog no. C7904-84C; US Biological, Swampscott, MA, USA) and either CMV-empty plasmid or CMV-Foxm1 expression plasmid, the latter of which lacks a 1–791 bp Foxm1 cDNA 5′ region to stabilize Foxm1 protein in the cell (Park *et al*., 2007). CMV-Renilla was used as an internal control to normalize transfection efficiency. Dual luciferase assay (Promega, Madison, WI, USA) was performed 24 h after transfection as described (Kim *et al*., 2005a, 2005b).

Nuclear extracts from siRNA-transfected A549 cells were cross-linked by addition of formaldehyde, sonicated and used for the immunoprecipitation with Foxm1 rabbit antiserum as previously described (Wang *et al*., 2005). Reverse cross-linked ChIP DNA samples were subjected to PCR using the oligonucleotides listed in Table 1. DNA binding was normalized to control ChIP DNA samples, which were immunoprecipitated using control rabbit serum.

Statistical analysis

We used Microsoft Excel program to calculate s.d. and statistically significant differences between samples using the Student's *t*-test. *P*-values <0.05 were considered statistically significant.

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